Metallic Nanoprobes for Enhanced Raman and Fluorescence Spectroscopy

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2003 Joint Service Scientific Conference on
Chemical and Biological Defense Research

18 November 2003

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE **18 NOV 2003**	2. REPORT TYPE **N/A**	3. DATES COVERED **-**
4. TITLE AND SUBTITLE **Metallic Nanoprobes for Enhanced Raman and Fluorescence Spectroscopy**		5a. CONTRACT NUMBER
		5b. GRANT NUMBER
		5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)		5d. PROJECT NUMBER
		5e. TASK NUMBER
		5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Physical Sciences Inc. 20 New England Business Center Andover, MA 01810**		8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)		10. SPONSOR/MONITOR'S ACRONYM(S)
		11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES
See also ADM001851, Proceedings of the 2003 Joint Service Scientific Conference on Chemical & Biological Defense Research, 17-20 November 2003. , The original document contains color images.

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**UU**	**23**	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std Z39-18

Essential Features of High Contrast Optical Bioassays

- Significant discrimination of analyte against multiple interferent species
- High sensitivity (single molecule)
- Operate in aqueous solution
- Reagents are produced using rational synthetic methods to ensure QC and ISO manufacturability
- Requires selective enhancement of analyte optical signal or high degree of multiplexing

Metallic Surfaces Modify Optical Processes

- **Metallic particles with subwavelength dimensions can increase emission intensity of fluorophores**
  - increase in radiative rate yields increased quantum yield (maximum enhancement factor = 1/QY)
  - **enhanced electromagnetic field between metallic nanoparticles** increases fluorescence emission cross-section by up to 10^5-fold;
  - decreased radiative lifetime yields higher photon flux and increased photostability
  - optimum particle diameter is 50 to 100 nm
  - optimum enhancement occurs in the region between ~50 Å (to avoid quenching) and ~200 Å from particle surface
- **Metallic particles with subwavelength dimensions can increase Raman scattering cross sections**
  - hot-spot surface-enhanced Raman scattering
  - **enhanced electromagnetic field between metallic nanoparticles** increases Raman cross-section in solution by up to 10^8-fold

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Metallic Nanoprobe Fabrication

- Current approaches include deposition of metal island films on substrates and salt-induced aggregation of metallic colloids
  - good enhancement is observed but reproducibility is a serious issue
  - variability in nanoprobe fabrication is responsible for assay variability
- Our approach is to synthesize metallic nanosphere structures with controlled interparticle spacing to exploit significant field enhancement between particles
  - start with simplest structure – nanosphere dimer
  - dimers permit fully solution-based assay

Assay Development Hierarchies

- **Three types of assay**
  - fluorophores
  - fluorescent tags
  - biomolecules (proteins)
- **Three levels of assay**
  - fluorophore and probe randomly interacting in solution
  - probes immobilized on surface randomly interacting with fluorophore in solution
  - fluorophore conjugated to probe

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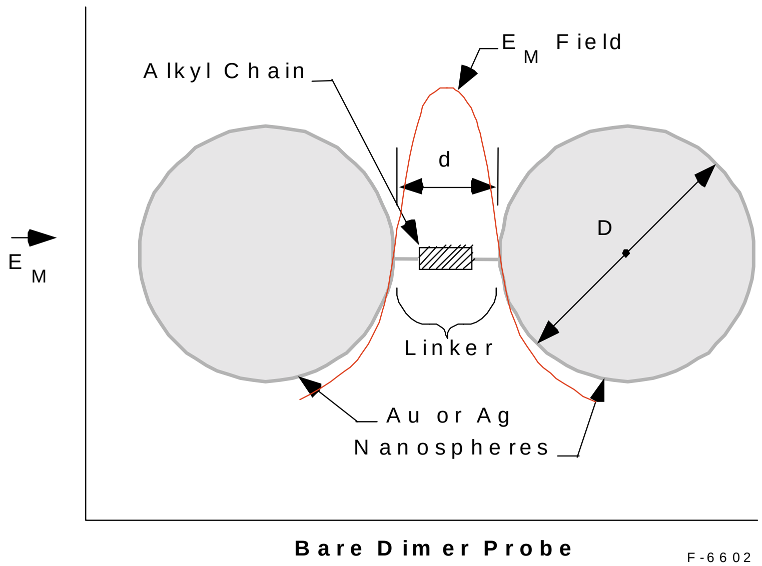
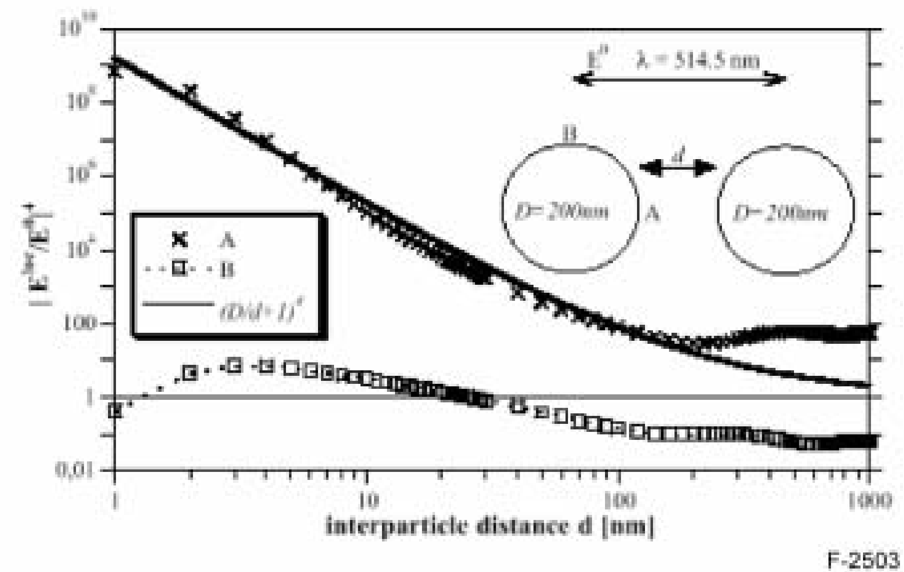
Schematic of Metallic Dimer Probe



Schematic of metallic dimer and its local electromagnetic field.



- Enhancement factor increases dramatically with decreasing D/d

- Maximum field enhancement factor ~ 10^3
- Fluorescence enhancement ~ $(E_M/E_o)^2 = 10^6$
- Raman enhancement ~ $(E_M/E_o)^4 = 10^{12}$

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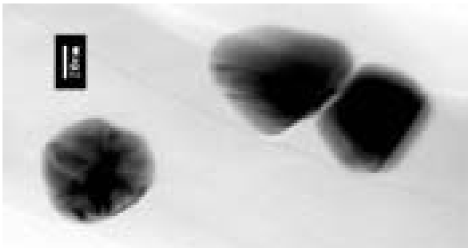
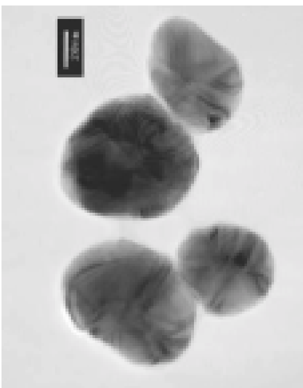
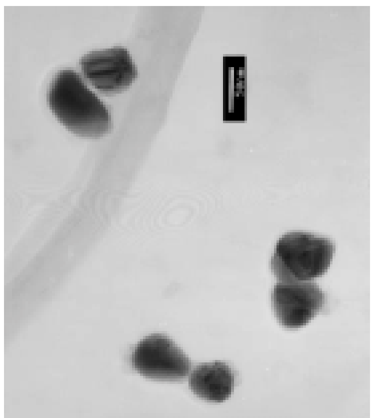
Synthesis of Metallic Dimer Probes







- Couple two metallic nanospheres together with an alkyldithiol linker to form dimer
  - vary Au/Ag sphere diameter from D = 20 to 80 nm
  - vary linker length d
  - vary intersphere spacing ratio from D/d = 5 to 75
- Dimer yield: ~80% (balance monomer + trimer) without purification
- Size exclusion chrmatography improves purity to >95%
- Highly reproducible
- Tradeoffs between control of EM field enhancement and interparticle volume available to analyte

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Synthesis of Metallic Dimer Probes

– 30 nm Ag colloid (British Biocell) is added to protected dithiol linker, then base is added to deprotect the thiol groups and produce the dimer

– obtain ~ 80% dimer yield (by TEM), with balance monomer and trimer

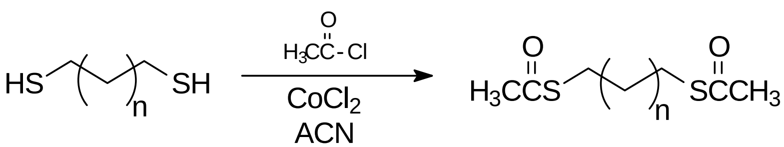


Figure 3. Synthetic route to protected linker molecules.

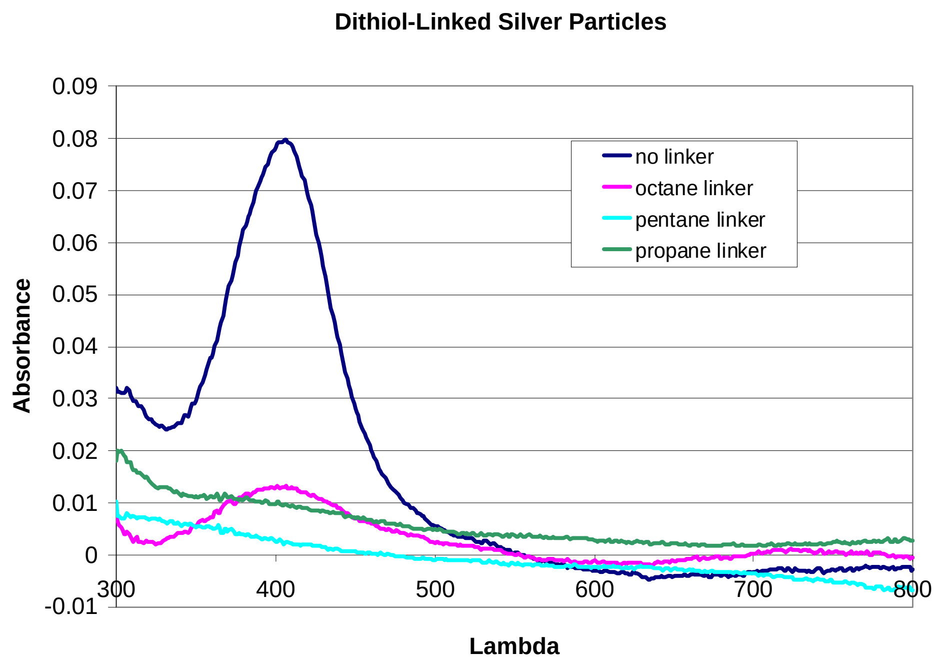
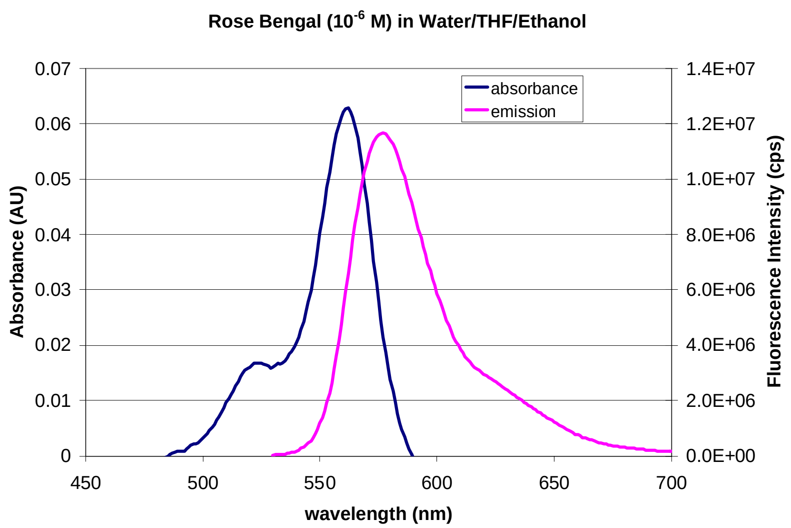
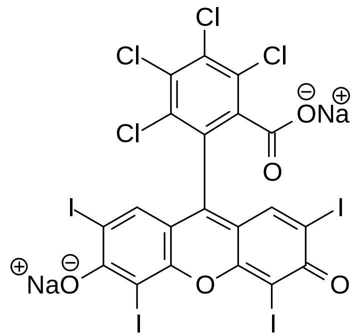


Figure 6. UV-vis absorption spectra of Ag monomers and dimers.

Characteristics of Rose Bengal Fluorophore





Molecular structure, absorbance and emission spectra of Rose Bengal in aqueous medium.

- Rose Bengal has modest quantum yield (~0.02 to 0.04)
- maximum enhancement due to increased radiative rate is 1/QY ~ 25 to 50

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Enhancement of Rose Bengal Fluorescence by Ag Nanoprobes in Solution

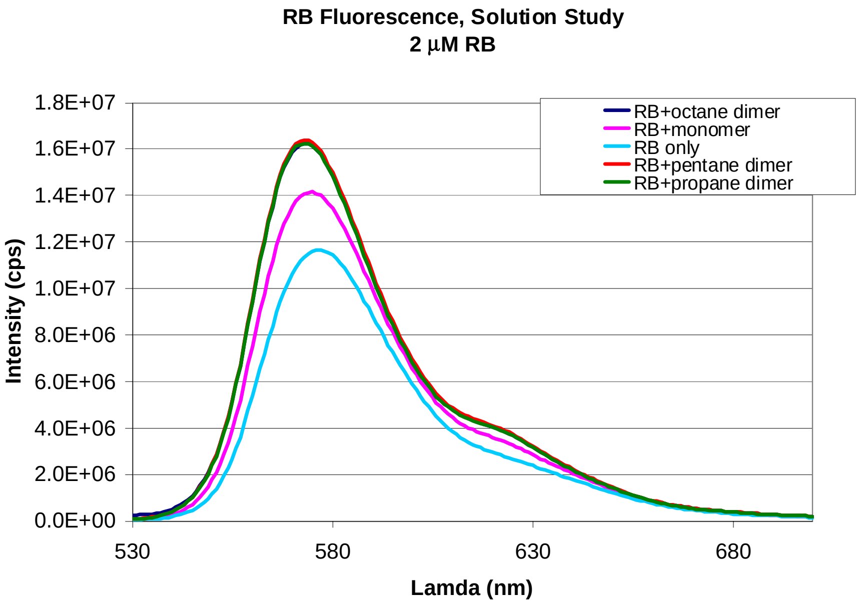

Figure 8. Fluorescence enhancement of RB in aqueous solution by free silver monomers and dimers.

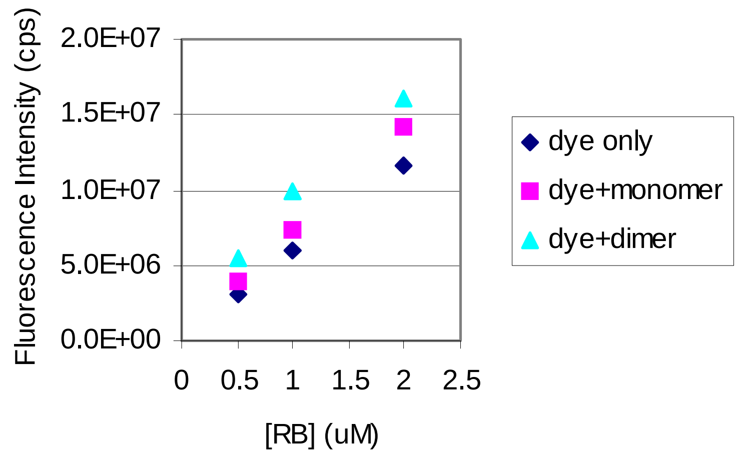

Figure 9. Dependence of RB fluorescence on RB concentration, for fixed monomer/dimer concentration.

- fluorophore to nanoparticle (monomer or dimer) ratio is 4000:1 (randomly mixed in solution)
- total observed emission is ensemble average over all fluorophores (enhanced and non-enhanced)
- in steady-state, only a small fraction of fluorophores are enhanced
- actual fluorescence enhancement is thus much greater
- dimer enhancement > monomer enhancement
- enhancement is independent of linker length

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Simple Enhancement Factor Model

The ratio of fluorescence intensities with (I_d) and without (I_o) dimers is given by

$$\frac{I_d}{I_0} \leq 1 + \frac{c_d N_A}{c_f}\left(\frac{R_f^A}{R_f}\right)$$

where

- c_f is the concentration of fluorophores in solution
- c_d is the concentration of metal dimers in solution
- R_f is the fluorescence radiative rate for the free fluorophores
- R_f^A is the fluorescence radiative rate for adsorbed fluorophores
- N_A is the saturation (max) number of fluorophores that can be adsorbed (and excited) on a dimer

For $N_A \sim 1$, $c_d = 0.5$ nM, and $c_f = 2\mu$M, the enhancement factor $R_f^A/R_f \geq 1500$.

$\rightarrow$ ***Ag metallic dimers generate >10^3-fold enhancements in fluorescent intensity***

$\rightarrow$ ***Ag monomers produce ~15-fold enhancement for Rose Bengal (theoretical max is 25 to 50)***

$\therefore$ Dimers produce larger fluorescence enhancement due to the presence of stronger EM fields localized in the interparticle region

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Enhancement of Rose Bengal Fluorescence by Surface-Immobilized Ag Nanoprobes

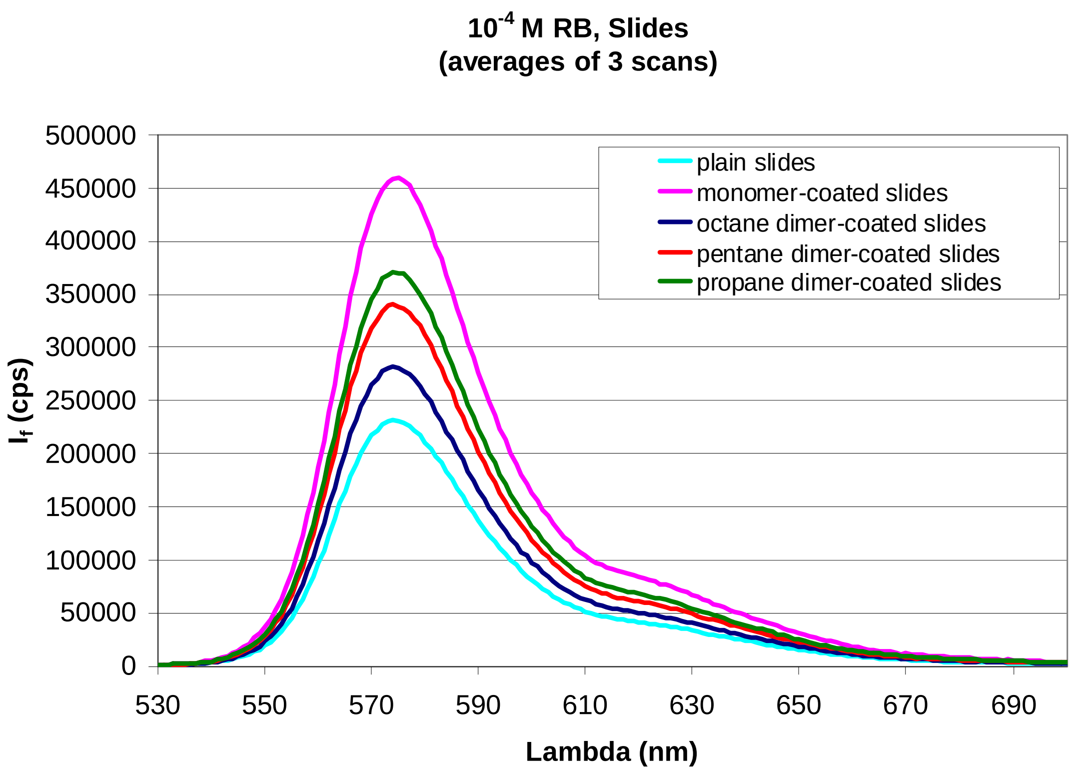

Figure 10. Fluorescence of RB on glass slides containing immobilized Ag monomers or dimers.

- Nanoprobes bound to thiolated glass slide
- Higher probe:fluorophore ratio produces greater observed emission enhancement
- Enhancement increases with decreasing linker length, in accordance with theory
- “Monomer” is likely an immobilized dimer with very small interparticle spacing

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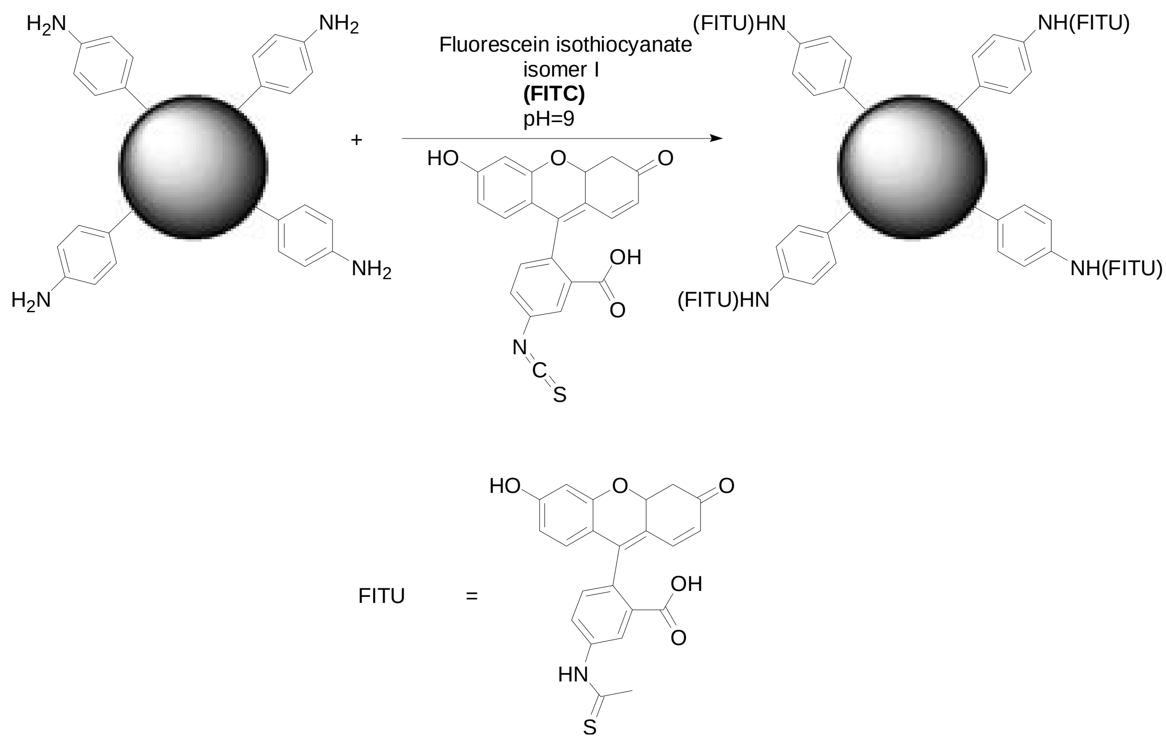
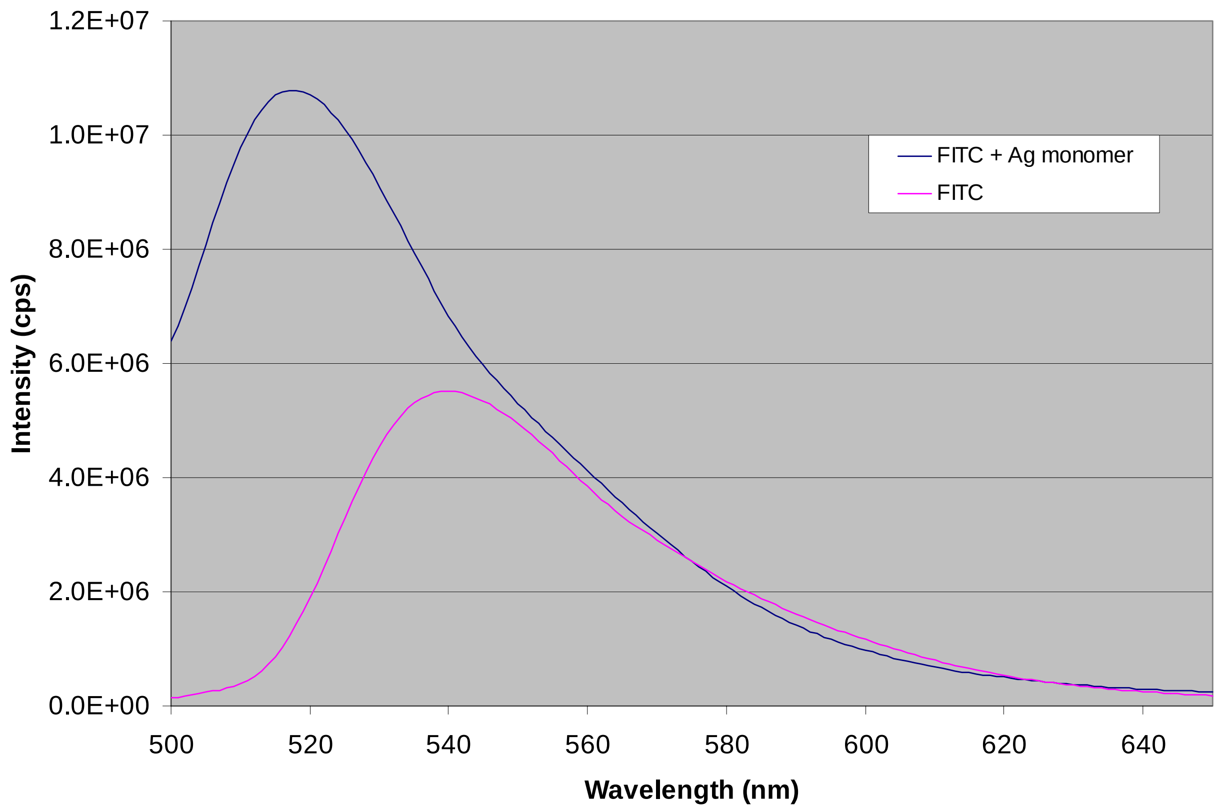
Conjugation of FITC to Ag Nanospheres



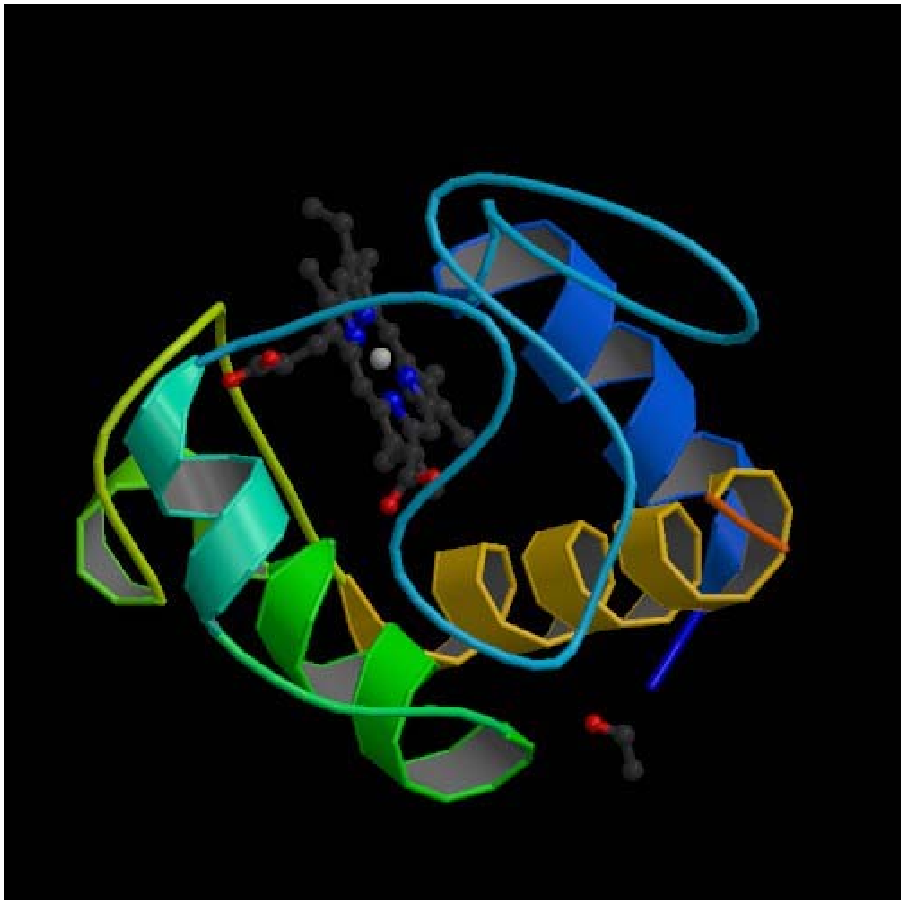
– FITC is a widely used label in molecular biology, assays

Conjugation of FITC to Ag Nanosphere Monomers Enhances Fluorescence in Solution



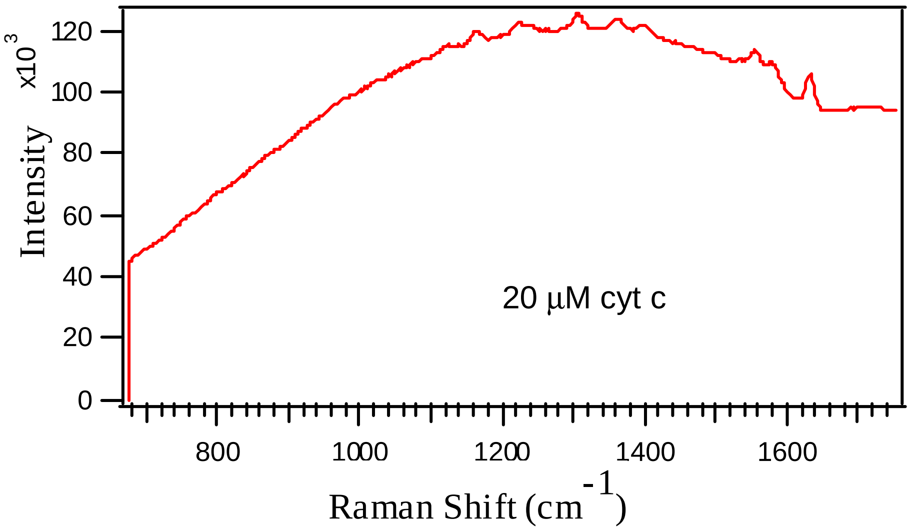
- 60 nM FITC (QY~0.8) and 1 nM nanosphere in H_2O; 488 nm excitation
- raw 2-fold fluorescence enhancement *for monomer*
- corrected for FITC/nanosphere ratio, the enhancement is ~100-fold
- nanosphere dimer expected to produce even larger enhancement

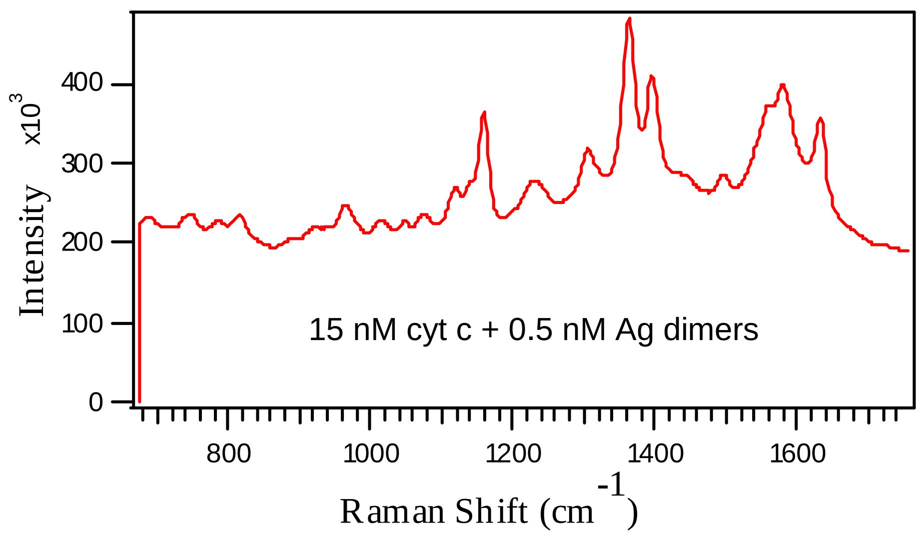
Properties of Cytochrome c



- Class of small electron-transfer proteins containing one or more heme groups
- Cyt-c release from mitochondria to the cytosol represents a critical step in apoptosis (programmed cell death) correlated to the activation of the caspase cascade
- Very short fluorescence emission lifetime (τ ~ 150 fs)
- Low quantum yield (~10^{-7})

Ag Dimer-enhanced Fluorescence of Cytochrome c





- bulk aqueous solution
- 514.5 nm excitation; red-shifted fluorescent emission
- observe raw 3200-fold fluorescence intensity enhancement
- corrected for cyt-c/dimer ratio, the enhancement is ~10^5-fold
- also observe ~10^8-fold enhancement in Raman scattering

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Dimer-enhanced Raman Scattering

- **Applied Ag dimer probe to SERS**
  - achieved 10^8-fold enhancement of SERRS of cytochrome-c in aqueous solution with Ag dimer (D/d~50)
  - dimer captures “hot-spot” enhancement observed in NaCl-induced Ag nanoparticle aggregates
  - dimer produces 30,000-fold greater enhancement than Ag monomer
  - total dimer-enhanced cross-section of cyt-c is $\sim 10^{-16}$ cm^2, equivalent to typical fluorophore
  - cyt-c detection limit is ~0.2 nM in solution
  - single-molecule detection of cyt-c with surface-immobilized dimer probes should be possible

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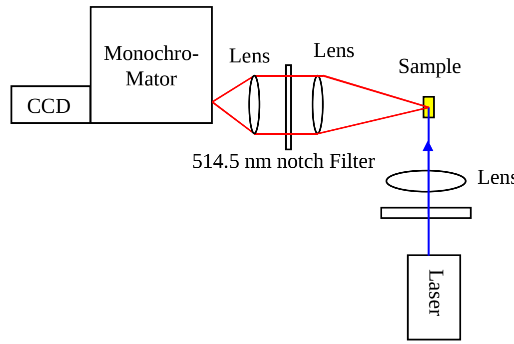
PSI Raman Scattering Setup



- The excitation wavelength is 514.5 nm (Ar+). The excitation power is 40 mW cw.
- A bandpass filter is used to block the laser plasma lines. A notch filter is used to block the Rayleigh light.
- A 1200 groove/mm grating is used to disperse the collected light.
- Integration Time is 10x60 sec (10 replicate spectra at 60 sec collection time per spectrum)

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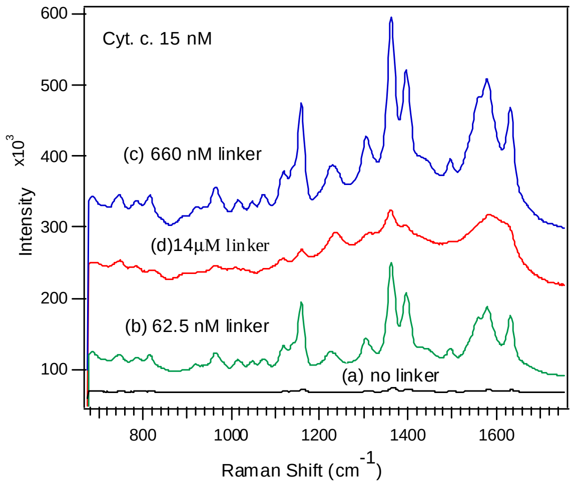
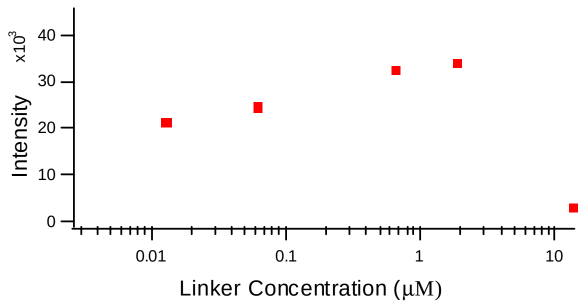
Effect of Alkyldithiol Linker on Cytochrome c SERS



SERS spectra of 15 nM cytochrome c solution. The 1,3 propanedithiol linker solution concentration is 0 nM, 62.5 nM, 660 nM, and 14 µM, respectively. The laser excitation power is 40 mW and 10 minutes integration time is used to collect the data.



Dependence of 1363 cm^{-1} peak intensity for cyt-c on the linker concentration. The cytochrome c concentration is fixed at 15 nM.

- 15 nM cytochrome c
- 0.5 nM Ag nanoparticles
- 1,3-propane dithiol linker
- optimal linker concentration is ~700 nM
- 514.5 nm excitation; 40 mW CW

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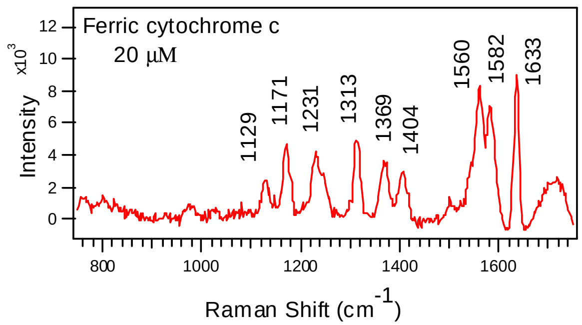
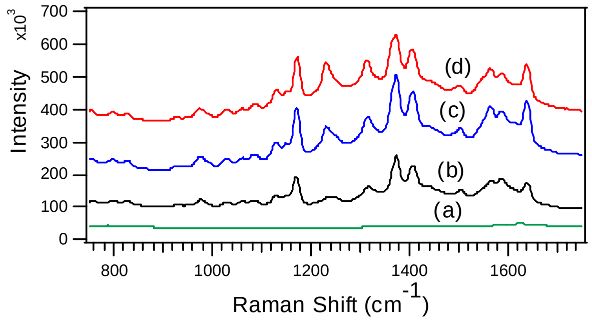
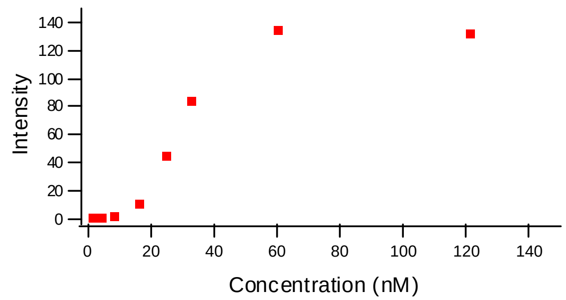
Concentration-dependence of Cytochrome c SERS





Dependence of Raman intensity on cyt-c concentration. (left) Bulk resonance Raman spectrum of 20 μM cyt-c in water; (right) SERS spectra of cyt-c and silver dimers in water. Cyt-c concentrations are (a) 1.65 nM ; (b) 33 nM; (c) 60 nM; and (d) 304 nM. The 1,3 propanedithiol linker concentration is 660 nM. The laser power is 40 mW and the integration time is 10 min**.**



Raman intensity of the 1363 cm^{-1} cyt-c peak as a function of cyt-c concentration in water. Laser power is 40 mW and the integration time is 10 min.

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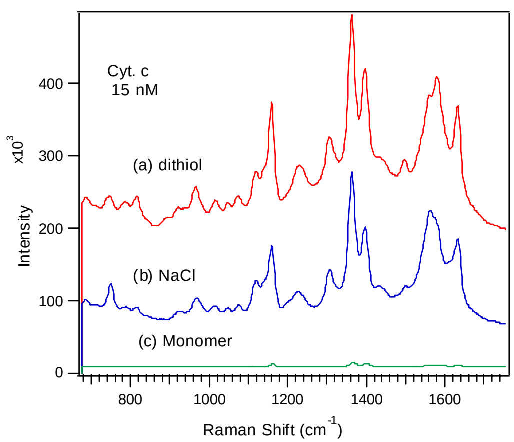
Ag Dimers Produce Greater Raman Enhancement of Cytochrome c than Aggregated Ag Colloids

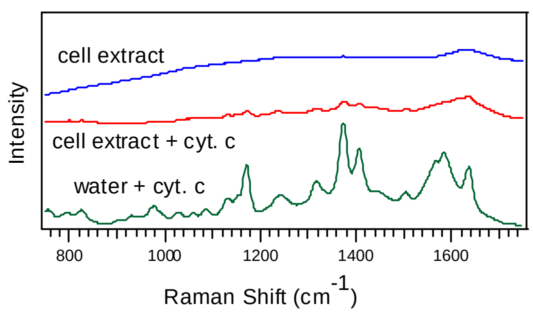


SERS spectra of 15 nM cyt-c in water obtained using Ag nanoparticle dimer (dithiol), 10 mM NaCl mixed colloid (NaCl), and colloid without aggregation agent (monomer).

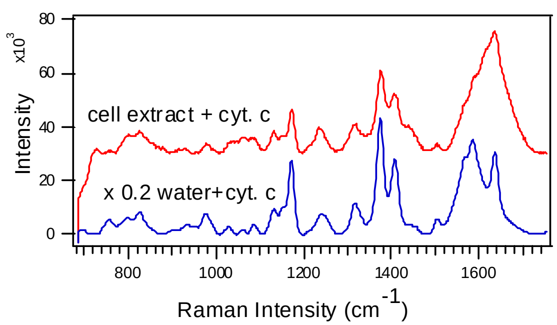
As a confirmatory experiment, we compared the SERS intensity for cyt-c generated with the Ag dimers to the intensity generated with a standard Ag nanoparticle colloid aggregated with NaCl. These colloidal aggregates contain random "hot spots" of high electromagnetic field enhancement. Note that the enhancement for the silver dimers is greater than that for the Ag colloid aggregate.

This result indicates that Ag nanoparticle dimers capture the essential features of the "hot spots" required for extreme Raman enhancement.

Ag Dimers Selectively Enhance Cytochrome c Raman in HeLa Cell Extract



Raman spectra of pure HeLa cell extract, extract+cyt-c, and cyt-c solution without cell extract.



Raman spectra of 15 nM cytochrome c mixed with cell extract and 25 nM Ag dimers. The spectrum of pure SERS of cytochrome c mixed with 25 nM Ag dimers is also shown (bottom curve) as a reference.

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Dimer Probes Can Incorporate Recognition/Binding Elements

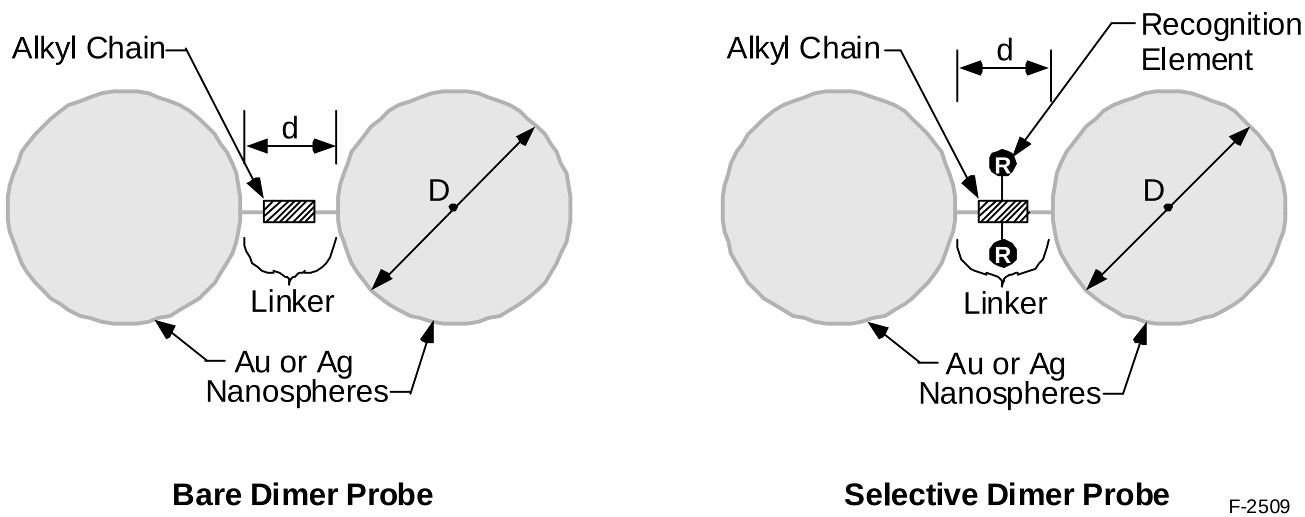

Figure 7. Schematic of dimer probes for single-molecule detection.